Phosphorylation of membrane proteins in response to temperature in an Antarctic *Pseudomonas syringae*

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Temperature-dependent phosphorylation and dephosphorylation of membrane proteins was studied *in vitro* in a number of psychrotrophic Antarctic bacteria which grow between 0 and 30 °C. One of them, a *Pseudomonas syringae* isolate, was studied in detail and was found to have three membrane proteins of molecular mass 30, 65 and 85 kDa which were phosphorylated differently in response to low and high temperatures. The 65 kDa protein was phosphorylated only at lower temperatures (between 0 and 15 °C). The 30 kDa protein was phosphorylated more at higher temperatures and was possibly a histidine kinase. This protein was present in all the psychrotrophic *Pseudomonas* species studied and in *Sphingobacterium antarcticus*. A possible role for these proteins in sensing environmental temperature is proposed.

Keywords: Antarctic psychrotrophs, *Pseudomonas syringae*, phosphorylation of proteins, temperature sensing

INTRODUCTION

Sensing environmental signals is of primary importance for adaptation in living systems. Bacteria, in general, use a 'two-component' regulatory system to achieve this (Bourret *et al.*, 1991; Stock *et al.*, 1989). In this system a 'sensor' molecule, usually a histidine kinase in the cell membrane, is activated by phosphorylation and the signal is transduced to the cytoplasmic 'regulator' protein by transphosphorylation of an aspartic acid residue. The regulator proteins generally act as transcription activators and therefore alter the pattern of gene expression. However, the sensing of environmental temperature, or thermosensory transduction, in bacteria remains little understood, although models have been proposed for mesophilic bacteria. An earlier study indicated the possible involvement of a chemosensory transducing pathway in thermosensory transduction in *Escherichia coli* (Maeda & Imae, 1979). More recently DnaK, a member of the heat-shock protein family of hsp70, was implicated as a cellular thermometer in *E. coli* (Craig & Gross, 1991; McCarty & Walker, 1991). The heat-shock sigma factor (σ^{32}) which regulates the expression of the heat-shock genes (Straus *et al.*, 1987, 1990) also controls the expression of DnaK. It is therefore likely that there exists a primary thermosensory transducing pathway which would interact with the specific sigma factor(s) and bring about expression of specific sets of genes so as to adapt to

higher or lower temperatures. In pathogenic yersiniae (*Yersinia pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*) the genes such as *lcrF* or the homologous *virF* have been implicated in temperature sensing, for virulence and pathogenesis (Hoe *et al.*, 1992; Hoe & Goguen, 1993). However, nothing is known about how psychrotrophic bacteria sense environmental temperatures.

In our studies with Antarctic micro-organisms (Jaganadham *et al.*, 1991; Ray & Shivaji, 1992; Ray *et al.*, 1989, 1991, 1992, 1994a, b; Shivaji *et al.*, 1988, 1989a, b, 1991, 1992; Chauhan & Shivaji, 1994) we observed that psychrotrophic bacteria could grow at temperatures between 0 and 30 °C and that their transcription and translation machinery was well adapted to function at 0 °C. Therefore, the Antarctic psychrotrophic bacteria could provide us with a unique model to study cold-adaptive features as well as adaptive responses to environmental temperatures. In one of the psychrotrophs, *Pseudomonas syringae*, the cold-inducible expression of certain genes and the existence of more than one sigma factor, including a cold-specific one, were also observed (unpublished observation). This indicated that differential expression of genes in response to temperature fluctuation (which is common in soil systems of continental Antarctica) could be the consequence of a primary temperature-sensing mechanism. Since the phosphorylation-dephosphorylation mechanism has been implicated in the primary

sensory transduction pathway of various environmental stimuli (Bourret *et al.*, 1991; Stock *et al.*, 1989), the temperature-dependent phosphorylation of membrane proteins was studied. It was observed that low temperature induced the phosphorylation of a 65 kDa membrane protein. Two other membrane proteins, of 30 and 85 kDa, were observed to be phosphorylated in response to higher temperature. Pulse-labelling of *P. syringae* cells with $^{32}\text{P}_i$ *in vivo* suggested that phosphorylation of these proteins could change in response to low and high temperature. Therefore, a possible role of these proteins in sensing temperature has been suggested.

METHODS

Bacterial strains and growth conditions. All the bacterial strains from soil samples of Antarctica were identified and maintained as previously reported (Shivaji *et al.*, 1989a). All the strains were psychrotrophic with an optimal growth temperature of about 22 °C. They were routinely grown in ABM (Antarctic bacterial medium, which contains bacto-peptone, 0.5%, w/v, and yeast extract, 0.2%, w/v) at room temperature (~22 °C) (Shivaji *et al.*, 1989a). The strains used in this study were *P. syringae* Lz4W, *P. fluorescens* 1W, *P. fluorescens* 9AW, *P. fluorescens* 39W (Shivaji *et al.*, 1989a), *Arthrobacter globiformis* 4B2W (Shivaji *et al.*, 1989b), *Micrococcus* sp. 30Y, *Planococcus* sp. Lz30R (Shivaji *et al.*, 1988) and *Sphingobacterium antarcticus* 6BY (Shivaji *et al.*, 1992).

Isolation of bacterial membranes and cytosol. The bacterial membranes were prepared by a method similar to that of Schnaitman (1971). Briefly, the cells were treated with lysozyme (60 µg ml⁻¹) in the membrane buffer (10 mM Tris, pH 8.0, 0.75 M sucrose, 2 mM EDTA and 1 µM PMSF) and sonicated for 2 min in a Branson sonifier in which the duty control switch was set at 40% and the output knob at 3 (power output of 70 W). The unbroken cells were removed by centrifugation at 8000 r.p.m. for 10 min in a Sorvall centrifuge. The total membrane fraction was pelleted by ultracentrifugation at 30000 r.p.m. for 2 h at 4 °C in a TL-100 table-top ultracentrifuge (Beckman). The supernatant was used as the cytosol fraction in this study. The inner membrane proteins were solubilized with 2% (v/v) Triton X-100 in the membrane buffer for 30 min at room temperature (~22 °C). After centrifugation at 30000 r.p.m. for 2 h, the supernatant was used as the inner membrane protein fraction. The outer membrane proteins were not solubilized under the above conditions (Schnaitman, 1971). For experimental purposes, the pellet of outer membrane was suspended in the membrane buffer and the suspension was used for phosphorylation assay.

Assay of phosphorylation activity. The phosphorylation assay *in vitro* was carried out with Triton X-100 solubilized membrane proteins. Briefly 30–40 µg of the membrane proteins were incubated in 10 mM Tris/HCl (pH 7.6) and 5 mM MgCl₂ for 10 min at 0, 5, 10, 15, 20, 22, 25 and 30 °C and the phosphorylation reaction was started with the addition of 20 nM [γ - ^{32}P]ATP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹). For assay of phosphorylation in presence of [γ - ^{32}P]GTP or [α - ^{32}P]ATP the equivalent amount, i.e. 20 nM (3000 Ci mmol⁻¹), of the respective nucleotide was used instead of [γ - ^{32}P]ATP. After 10 min an equal volume of 2 × sample buffer was added, boiled for 2 min, cooled and analysed on an SDS-polyacrylamide gel (Laemmli, 1970). The phosphorylated proteins were visualized by autoradiography. For quantification, the radioactive bands on the dried gel (identified with the help of the

corresponding autoradiogram) were cut out and the associated radioactivity was measured directly (dry count) in a scintillation counter. It is to be noted that the average delay between the actual day of the experiment and the counting of radioactivity of the gel fragments was about 5–6 d. Therefore the radioactivity data shown in Figs 2 and 3 are less than the actual incorporation. Also to be noted is the fact that the efficiency of a dry count is less than that in the presence of a liquid scintillant. Nevertheless the data presented in the figures are proportionate to the intensity of the bands seen in the autoradiograms.

Phosphorylation study *in vivo*. For *in vivo* phosphorylation, *P. syringae* cells (OD₆₀₀ 0.6) were pulsed in the presence of ^{32}P -labelled orthophosphate (35 µCi per ml culture) for 30 min at 4 °C, or for 15 min at 22 °C. The cells were pelleted, washed and transferred into fresh ABM medium which was preincubated at the desired temperature. At the respective time points the cells were pelleted by centrifugation either at 22 °C or at 4 °C and lysed in the SDS sample buffer of Laemmli (1970) by boiling for 2–5 min and analysed on an SDS-polyacrylamide gel. During labelling with [γ - ^{32}P]ATP, the cells were incubated in ABM medium in the presence of 100 µCi labelled nucleotide for 30 min to 1 h and processed as above. It is to be noted that the temperatures chosen for phosphorylation experiments were 4 °C (for lower temperature) and 22 °C (for higher temperature), unlike most of the *in vitro* experiments, where 0 and 30 °C were used. This was because 30 °C was the maximum growth temperature for the psychrotrophs and could have caused unwanted stress to the cells. The selection of 4 °C as the lower temperature for *in vivo* phosphorylation was simply due to the fact that growing cells at 4 °C was easier.

Acid and alkali stability of proteins and analysis of phosphoamino acids. Acid and alkali stability of the phosphorylated proteins was studied at 50 °C for 10 min or at 37 °C for 20 min in the presence of 0.1 M HCl and 0.1 M NaOH respectively, according to the method of Mukai *et al.* (1990). For analysis of the phosphoamino acid, the *in vitro* ^{32}P -labelled phosphorylated proteins were transferred on to Immobilon-P membrane (Millipore) by the method of Kyhse-Anderson (1984). After visualizing the protein bands following autoradiography, the band corresponding to the 30 kDa protein was cut out and the protein in the membrane strip was hydrolysed in 3 M KOH, neutralized and analysed by paper chromatography as described by Smith *et al.* (1978).

RESULTS AND DISCUSSION

Phosphorylation of membrane-associated proteins *in vitro*

Membrane-associated proteins are expected to be likely candidates for sensing environmental conditions. Therefore to localize the protein which was phosphorylated following cold treatment, the total cell membranes of *P. syringae* were phosphorylated in the presence of [γ - ^{32}P]ATP. SDS-PAGE analysis and subsequent autoradiography revealed that a 65 kDa protein was phosphorylated specifically at 0 °C. The corresponding protein band was not visible in the Coomassie-Blue-stained gel, indicating that the protein was present in a minute quantity. Temperature dependence of the cold-induced labelling of the 65 kDa proteins suggested that the phosphorylation takes place between 0 and 15 °C (data not shown). Above 20 °C the 65 kDa protein was not

Fig. 1. Phosphorylation of outer and inner membrane proteins of *P. syringae*. (a) Phosphorylated proteins of outer membrane at 0 °C (lane 1) and at 30 °C (lane 2) and of inner membrane at 0 °C (lane 3) and at 30 °C (lane 4). The autoradiogram was developed after 6 h. The 85 kDa band is not clearly visible in this figure. (b) Triton X-100 solubilized membrane fraction was phosphorylated in the presence of either [γ - 32 P]ATP or [γ - 32 P]GTP at 0 °C or 30 °C for 10 min and analysed by SDS-PAGE and autoradiography. Lane 1, [γ - 32 P]ATP, 0 °C; lane 2, [γ - 32 P]ATP, 30 °C; lane 3, [γ - 32 P]GTP, 0 °C; lane 4, [γ - 32 P]GTP, 30 °C. All the phosphorylated protein bands including the 85 kDa band are distinctly visible.

phosphorylated but two other proteins (30 and 85 kDa) were observed to be phosphorylated more at higher temperatures (e.g. at 30 °C) in the inner membrane (Fig. 1a, b). Triton X-100 solubilization of the total membrane (Schnaitman, 1971) indicated that all three proteins were associated with the inner membrane (Fig. 1a). The few phosphorylated bands seen to be associated with the outer membrane (Fig. 1a, lane 2) were probably due to phosphorylation of lipopolysaccharides rather than proteins (unpublished observation; Ray *et al.*, 1994b).

While both [γ - 32 P]ATP and [γ - 32 P]GTP could act as phosphate donors for the phosphorylation of the three proteins, [γ - 32 P]GTP acted as a better phosphate donor for the 30 kDa protein (Fig. 1b). In contrast, [γ - 32 P]ATP acted as a better donor for phosphorylation of the 65 kDa protein. Solubilization of the phosphorylated reaction mixture in Laemmli sample buffer at 25 and 100 °C did not alter the mobility of the phosphoproteins in the SDS-polyacrylamide gels (data not shown). Therefore, it is likely that the three phosphorylated proteins represent three different species, rather than the dimer or oligomer of the 30 kDa protein. It is also to be noted that [α - 32 P]ATP could not phosphorylate these proteins, ruling out the possibility that the observed phosphorylation was due to adenylation (data not shown).

Time kinetics of *in vitro* phosphorylation of the three proteins (Fig. 2) indicated that at 30 °C the 30 kDa protein was phosphorylated to a maximum extent within 15 s and reached a plateau value by 2 min but subsequently reduced to a basal level after 30 min (data not shown). However,

Fig. 2. Kinetics of phosphorylation of the membrane proteins of *P. syringae*. Triton X-100 solubilized membrane proteins were phosphorylated in the presence of [γ - 32 P]ATP at 0 and at 30 °C for various periods (15 s to 15 min) and resolved by SDS-PAGE. After visualization of the phosphorylated protein bands by autoradiography, the bands were cut from the gel and counted directly (dry count) in a liquid scintillation counter. The counts associated with the 30 kDa (●) and 85 kDa (▲) proteins were plotted from the kinetics of the 30 °C reaction, and the 65 kDa (○) protein counts were from the 0 °C reaction mixture. The inset shows the results of one such analysis. The bands corresponding to the 30 kDa protein (p30) and the 85 kDa protein (p85) were exposed for 16 h and the band corresponding to the 65 kDa protein (p65) was exposed for 30 h. The results are from the same gel.

in contrast, the 85 kDa protein at 30 °C was phosphorylated only after 5 min and the phosphorylation steadily increased, reaching a plateau after 15–20 min. The low temperature (0 °C) induced phosphorylation of the 65 kDa protein was observed after 1 min, reaching a maximum after 15–30 min. The fate of these three proteins following phosphorylation at 0 or 30 °C and subsequent temperature shift to 30 or 0 °C was also monitored. When the phosphorylation reaction was carried out at 0 °C in the presence of [γ - 32 P]ATP and then shifted to 30 °C, the intensity of the 65 and 85 kDa phosphorylated proteins showed a time-dependent inverse kinetic relationship (Fig. 3a, b). Further, the 30 kDa protein under similar temperature-shift conditions exhibited increased phosphorylation immediately after the temperature shift but remained more or less unchanged afterwards. Interestingly, in the reverse temperature-shift experiment (30 to 0 °C), the status of phosphorylation of the 30 and 85 kDa proteins did not alter significantly (Fig. 3a, c) but the 65 kDa protein, which was not phosphorylated at 30 °C, appeared to become phosphorylated slowly, reach-

Fig. 3. Kinetics of phosphorylation of the membrane proteins of *P. syringae*. (a) Triton X-100 solubilized membrane proteins were phosphorylated in the presence of [γ - ^{32}P]ATP for 10 min at 0 and 30 °C and then transferred to 30 and 0 °C, respectively. After transfer, equal aliquots were collected from the tube at various time points (0 to 60 min) and analysed by SDS-PAGE and autoradiography. Lanes 1 to 6 are the products of transfer from 0 to 30 °C at 0, 5, 10, 15, 30 and 60 min, respectively. Lanes 7 to 12 are the products of transfer from 30 to 0 °C at the same time intervals, respectively. (b, c) After autoradiographic visualization of the protein bands, the gel pieces corresponding to each labelled band were cut out and the radioactivity was measured directly (dry count) in a liquid scintillation counter. (b) Results of the 0 to 30 °C transfer reaction. (c) Results of the 30 to 0 °C transfer reaction. ●, 30 kDa protein; ○, 65 kDa protein; ▲, 85 kDa protein. The points are the means of the data from two gels.

ing a maximum after 30 min. It is to be noted here that in these experiments the unincorporated [γ - ^{32}P]ATP molecules were not separated from the reaction mixture. Following separation of the proteins from unincorporated [γ - ^{32}P]ATP by passing through a column (NucTrap probe purification column, Stratagene), it was found that the other proteins whose phosphorylation was temperature (high or low) dependent did not become labelled on temperature-shift. This indicated that the change of phosphorylation pattern was not due to a phosphotransfer reaction but due to separate kinase reactions. Although the actual relationship of phosphorylation-dephosphorylation between these proteins could not be established in these experiments, it could be argued that the phosphorylation status of these three proteins was modulated by temperature, and hence may enable the bacterium to sense the environmental temperatures. However, in some experiments during 30 to 0 °C shift (Fig. 3a) the 85 kDa protein showed a slight increase in phosphorylation. The reason for this is not clear.

Phosphorylation of proteins in cells *in vivo*

Experiments with intact cells incubated in the presence of carrier-free ^{32}P -labelled orthophosphate or with [γ - ^{32}P]ATP (Fig. 4a) also indicated that membrane proteins were phosphorylated. At 4 °C, phosphorylation of the 65 kDa protein in *P. syringae* was observed. However, this temperature-dependent phosphorylation does not necessarily establish that the cells use this as a mechanism to sense temperature. Therefore, an attempt was made to pulse-label the cells with $^{32}\text{P}_i$ in ABM medium and the phosphorylation of proteins was studied following a shift of temperature (Fig. 4b). The results indicate that when the cells were pulse-labelled for 15 min at room temperature (~ 22 °C) and then transferred to 4 °C they did not show much alteration in the radioactive intensity of the 30 kDa protein up to 1 h. However, the 30 kDa band increased in intensity within 5 min after the culture temperature was raised back to 22 °C. The protein band again lost its intensity after 1 h when the temperature was reduced to 4 °C. This repeated dynamic change of radioactivity of the 30 kDa band reflects a strong correlation of phosphorylation-dephosphorylation state with a shift of temperature, and therefore implies a possible thermosensing role. The cold-induced phosphorylated protein of 65 kDa, however, was not as prominent as the 30 kDa protein in this *in vivo* pulse-chase experiment. Moreover, the 65 kDa region of the gel is masked by intense non-specific background radioactivity due to labelled lipopolysaccharides and other unknown materials (unpublished observation). The higher-temperature-induced 85 kDa protein was also noticeable in the experiment (Fig. 4b). It is important to note that in these experiments distinction could not be made between membrane and cytosolic proteins and the assignment of the protein is based on their molecular masses and acid- and alkali-stability (Fig. 4c). However, after a longer exposure (about 3 d or more) a greater number of proteins were visible along with a high background which might contain the labelled cytosolic proteins.

Fig. 4. *In vivo* phosphorylation of *P. syringae* cells. (a) Cells were labelled with [γ - 32 P]ATP at 4 °C (lane 1) and at 22 °C (lane 2). The samples were analysed by 10% SDS-PAGE and visualized by autoradiography. (b) Pulse-labelling experiments of *P. syringae* cells with 32 P_i. The growing cells were pulsed with 32 P_i for 15 min at 22 °C (lane 1), washed and then transferred to 4 °C for 15 min (lane 2), 30 min (lane 3) and 1 h (lane 4). The cells were again transferred back to 22 °C for 5 min (lane 5), 15 min (lane 6), 30 min (lane 7) and 1 h (lane 8) and retransferred to 4 °C for 15 min (lane 9) and 1 h (lane 10). The samples were lysed directly in SDS sample buffer by boiling for 2 min and analysed on a 10% SDS-PAGE and by autoradiography. (c) Acid- and alkali-stability of *in vivo* 32 P-labelled proteins. Lane 1, cells were directly analysed after 32 P labelling at 22 °C; SDS-lysed cells were incubated in 0.1 M NaOH (lane 2), 0.1 M HCl (lane 3) or 0.1 M Tris/HCl, pH 7.5 (lane 4) before analysis on 10% SDS-PAGE.

The phosphorylation of cytoplasmic membrane proteins by [γ - 32 P]ATP in intact cells is interesting since [γ - 32 P]ATP cannot normally penetrate cells and hence it is likely that the temperature sensing proteins (65 and 30 kDa) are probably accessible from the periplasmic side of the cells. Another possible explanation could be that the [γ - 32 P]ATP was broken down into its constituents, and the 32 P_i thus liberated re-entered the cytoplasmic nucleotide pool and phosphorylated the proteins.

Nature of phosphorylation and phosphorylated proteins

The acid- and alkali-stability of phosphate groups of a protein generally provide a preliminary indication of the type of bond by which the phosphate groups are attached to the amino acids and thereby also suggest the nature of amino acids which are phosphorylated (Smith, 1984; Mukai *et al.*, 1990; Roy Chowdhury *et al.*, 1992). While *N*-linked phosphates are acid-labile but alkali-stable, the acyl phosphates are both acid- and alkali-labile. Among *O*-linked phosphates, tyrosine phosphates are both acid- and alkali-stable whereas serine and threonine phosphates are acid-stable but alkali-labile (Martensen, 1984; Fujitaki & Smith, 1984). Therefore acid- and alkali-stability of the phosphorylated membrane proteins of *P. syringae* was studied. Table 1 and Fig. 5(a) summarize the acid and alkali lability of the various phosphorylated proteins from the membrane. The data indicate that the 30 kDa mem-

Table 1. Acid- and alkali-stability of the temperature-modulated phosphorylated proteins of *P. syringae*

Stability was determined in 0.1 M HCl or 0.1 M NaOH at 50 °C as described in Methods. —, Not stable; +, stable.

Temperature	Protein	Stability	
		Acid	Alkali
Low (0 °C)	65 kDa	—	—
	30 kDa	—	+
	85 kDa	+	+

brane protein was probably phosphorylated at the histidyl residue. Phosphoamino acid analysis (Fujitaki & Smith, 1978) of the alkali hydrolysate of the 30 kDa protein upon paper chromatography confirmed that the protein was indeed phosphorylated at the histidyl residue (Fig. 5b). The kinetics of phosphorylation (Fig. 2) suggested that it was probably a histidine kinase since it is phosphorylated within 15 s of addition of [γ - 32 P]ATP or GTP to the kinase reaction mixture.

The phosphoamino acid in the membrane-bound 85 kDa protein was both acid- and alkali-stable, indicating that it could be a phosphotyrosine. However, due to the small amount of radioactivity in the protein, direct phos-

Fig. 5. Nature of phosphoamino acids in the phosphorylated proteins. (a) Acid- and alkali-stability of the phosphorylated proteins. The phosphorylated proteins were incubated in 0.1 M Tris/HCl, pH 7.5 (lane 1), 0.1 M NaOH (lane 2) or 0.1 M HCl (lane 3) for 20 min at 37 °C following a phosphorylation reaction of the total membrane protein in the presence of [γ - 32 P]ATP at 0 °C. The proteins were then subjected to 10% SDS-PAGE and autoradiography. (b) Phosphoamino acid analysis of the 30 kDa membrane protein. The analysis was done by the method of Smith *et al.* (1978). The origin, solvent front and the position of P-Ser are marked. The positions of P-Lys and P-His have been marked from the R_f values given by Smith *et al.* (1978).

phoamino acid analysis was not possible. The acid- and alkali-lability of the cold-induced phosphorylated 65 kDa protein indicated that it was probably due to acyl phosphate. However, this protein retains its phosphate group during usual staining and destaining in presence of 7.5% acetic acid and therefore the exact nature of this phosphoamino acid could not be confirmed. It is also interesting to note that the 30 kDa histidine phosphorylated protein did not lose its phosphate during standard staining–destaining of the gel in the presence of acetic acid, following SDS-PAGE. However, the protein lost its phosphate in 0.1 M HCl even after 15 min at 37 °C. The reason for this unusual stability of the protein in presence of acetic acid is not clear.

Temperature sensing in psychrotrophic bacteria

Sensing of temperature is an important aspect of adaptation of psychrotrophs to fluctuating environmental conditions. In this respect, the Antarctic psychrotrophic *P. syringae* has probably evolved a class of membrane proteins which can directly sense the external temperature by phosphorylation and dephosphorylation. The sensing of temperature in mesophiles such as *E. coli* by the proteins DnaK (McCarty & Walker, 1991) and σ^{32} (Straus *et al.*, 1987), and in pathogenic bacteria such as *Yersinia pestis* by the activator protein LcrF (Hoe & Goguen, 1993), may also be mediated through a similar modification of the membrane proteins which are yet to be identified. The possible involvement of the chemosensory proteins of the membrane in the sensing of temperature

Fig. 6. *In vitro* phosphorylation of membrane proteins from various Antarctic psychrotrophs. Triton X-100 solubilized membrane proteins were phosphorylated in the presence of [γ - 32 P]ATP and analysed by SDS-PAGE and autoradiography. Phosphorylated proteins at, respectively, 0 °C and 30 °C of: *P. fluorescens* 1W (lanes 1 and 2), *P. fluorescens* 9AW (lanes 3 and 4), *P. fluorescens* 39W (lanes 5 and 6), *S. antarcticus* 6BY (lanes 7 and 8), *Micrococcus* sp. 30Y (lanes 9 and 10), *Planococcus* sp. Lz3R (lanes 11 and 12) and *A. globiformis* 4B2W (lanes 13 and 14).

(Maeda & Imae, 1979) in *E. coli* would require a more detailed study. Our proposed studies involving the purification of the membrane proteins and cloning of the possible ‘sensor’ and the ‘regulator’ genes by PCR amplification of conserved regions of these genes may shed some light on the thermosensory transduction in *P. syringae*. However, the question as to whether other psychrotrophic bacteria from Antarctica also sense environmental temperature by similar mechanisms remains unanswered. In this context, we studied the phosphorylation of membrane proteins from these bacteria in response to low (0 °C) and high (30 °C) temperatures *in vitro* in the presence of [γ - 32 P]ATP (Fig. 6). The results indicated that all representatives of the genera *Pseudomonas* and the newly identified species *Spingobacterium antarcticus* (Shivaji *et al.*, 1992) contained the 30 kDa phosphorylated protein which is possibly a histidine kinase. The species belonging to the genera *Micrococcus*, *Planococcus* and *Arthrobacter* did not contain this protein but did contain different sets of proteins (Fig. 6) which were phosphorylated differently in response to low and high temperatures. Therefore, it is likely that various genera of bacteria may have evolved different sets of proteins to sense the environmental temperature. A note of caution must be added in respect of our postulate that the psychrotrophic bacteria sense environmental temperatures by phosphorylation–dephosphorylation of membrane and/or cytoplasmic proteins. Our study only indicates a correlation of phosphorylation–dephosphorylation events with the experimental temperatures. The results could as well be interpreted as involvement of the proteins in temperature-dependent metabolic activities of the cells, such as membrane transport and other similar processes. In summary, various psychrotrophic bacteria of Antarc-

tica show temperature-dependent phosphorylation of membrane proteins. The molecular masses of the phosphorylated proteins among the various bacteria vary. One of the bacteria, a *P. syringae* isolate, which was studied in detail exhibited a specific set of membrane proteins (30, 65 and 85 kDa) which were phosphorylated differently in response to low and high temperatures. These proteins might be responsible for primary sensing of environmental temperature, being transmitted through the cytoplasm for further propagation of the signal.

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